α1,4-GALACTOSYLTRANSFERASE AND DNA ENCODING THEREOF

BACKGROUND OF THE INVENTION

1. Field of the Invention

The present invention relates to the production of a polypeptide using a recombinant DNA, or to a tool useful for diagnosis or treatment of diseases, or more specifically to α1,4-galactosyltransferase and DNA encoding thereof, a recombination vector containing the DNA, and a transformed cell transfected with the DNA or by the recombination vector, and to a method for producing Gb3/CD77 or globo-series glycolipids by using the transformed cell.

2. Description of the Related Art

Glycosphingolipids are amphipathic molecules(ref.1) that are synthesized by sequential actions of glycosyltransferases(ref.2). Addition of one of three different sugars onto lactosylceramide (which may be termed "LacCer" hereinafter) results in the synthesis of either one of three major glycolipid series, i.e., ganglioside-series (α 2,3-sialic acid), lacto/ neolacto-series (β1,4-*N*-acetylglucosamine) and globo-series (α1,4-galactose). Although a number of genes coding for enzymes responsible for the synthesis of the carbohydrate moiety of glycosphingolipids have been recently isolated(ref.3), no glycosyltransferase genes specific for the synthesis of globo-series glycolipids have been

isolated to date.

Globotriaosylceramide (hereinafter sometimes referred to as "Gb3") is synthesized by α1,4-galactosyltransferase (α1,4 Gal-T) from LacCer(ref.4). This glycolipid has been characterized on red blood cells as the P^k antigen of the P blood group system(ref.5). Since Wiels et al(ref.6) reported that Gb3 was a Burkitt's lymphoma associated antigen, the expression and biological significance of Gb3 have been vigorously studied(ref.7, 8 and 9). Since Gb3 was clustered as CD77, this antigen will be referred to as Gb3/CD77.

Gb3/CD77 was reported to be expressed in high amounts on Burkitt's lymphoma cells. However, it is now considered to be a differentiation antigen expressed on B cells, and can also be found in some malignant tumors of B cell lineage(ref.7). Among normal leukocytes, it is only expressed on a subset of tonsillar B cells in the germinal centers (GC)(ref.9). Interestingly, GC B lymphocytes expressing Gb3/CD77 undergo rapid and spontaneous apoptosis when isolated and cultured *in vitro*(ref.11). Furthermore, Burkitt's lymphoma cells with Gb3/CD77 antigen were also easily induced to enter apoptosis upon culture at low serum concentration or cross-linking by anti-immunoglobulin M antibodies(ref.12).

Gb3/CD77 has been recognized as a receptor for verotoxins(VTs), the Shiga-like toxin from *E. coli* O157 strain that can trigger serious cytotoxic effects(ref.13 and 14). VT B-subunit specifically binds to Gb3/CD77, then A subunit is incorporated into cells, resulting in the degradation of 28 S ribosomal RNA and cell death(ref.15).

However, only B-subunit is also able to induce apoptosis when cross-linked(ref.16). These results indicate that Gb3/CD77 is a critical molecule in mediating apoptosis signals, although the precise mechanisms remain to be investigated.

As stated above, it was revealed that Gb3/CD77, a globo-series glycolipid, is syntheized by addition of α1,4-galactose to LacCer(ref.4), but the glycosyltransferase specific for this synethetic reaction has not been isolated yet. An object of the present invention is to isolate a Gb3/CD77 synthase, that is, α 1,4-galactosyltransferase, and DNA encoding thereof, and to provide the use thereof.

The present inventors have studied hard to achieve the above objects, and succeeded in isolating DNA encoding α1,4-galactosyltransferase, revealing its nucleotide sequence, and confirming that the DNA is responsible for the expression of active α1,4-galactosyltransferase. Thus, they achieved the present invention.

SUMMARY OF THE INVENTION

The present invention provides a polypeptide of (a) or (b) below (hereinafter sometimes referred to as "the polypeptide of the present invention"):

(a) a polypeptide consisting of an amino acid sequence represented by the amino acid Nos. 46-353 in SEQ ID NO: 2; or

(b) a polypeptide which comprises an amino acid sequence

including substitution, deletion, insertion or transposition of one or few amino acids in the amino acid sequence of (a) and which has an enzymatic activity to transfer a galactose residue from a galactose donor to C4 position of galactose residue of lactosylceramide or galactosylceramide which serves as an acceptor.

The polypeptide of the present invention also includes a polypeptide of(a′) or (b′) below:

(a′) a polypeptide consisting of an amino acid sequence represented by the amino acid Nos. 20-353 in SEQ ID NO:2; or

(b′) a polypeptide which comprises an amino acid sequence including substitution, deletion, insertion or transposition of one or few amino acids in the amino acid sequence of (a′) and which has an enzymatic activity to transfer a galactose residue from a galactose donor to C4 position of galactose residue of lactosylceramide or galactosylceramide which serves as an acceptor.

The polypeptide of the present invention also includes a polypeptide of(a″) or (b″) below:

(a″) a polypeptide consisting of an amino acid sequence represented by SEQ ID NO:2; or

(b″) a polypeptide which comprises an amino acid sequence including substitution, deletion, insertion or transposition of one or few amino acids in the amino acid sequence of (a″) and which has an enzymatic activity to transfer a galactose residue from a galactose donor to C4 position of galactose residue of lactosylceramide or galactosylceramide which serves as an acceptor.

The present invention also provides a DNA encoding the polypeptides according to any one of above polypeptides(hereinafter sometimes referred to as "the DNA of the present invention"). The DNA of the present invention includes a DNA represented by (a) or (b) below:

(a) a DNA comprising a nucleotide sequence represented by nucleotide Nos. 269 to 1192 in SEQ ID NO:1; or

(b) a DNA hybridizable with a DNA comprising a nucleotide sequence represented by SEQ ID NO:1, a nucleotide sequence complimentary to SEQ ID NO:1, or a part of those sequences, under a stringent condition.

The DNA of the present invention also includes a DNA encoding a polypeptide having an enzymatic activity to transfer a galactose residue from a galactose donor to C4 position of galactose residue of lactosylceramide or galactosylceramide which serves as an acceptor.

The present invention still further provides a recombination vector containing the DNA of the present invention.

The present invention still further provides a transformed cell obtained by transfecting a host cell with the DNA of the present invention or the recombination vector.

The present invention still further provides a method for producing the polypeptide of the present invention, comprising the steps of:

producing the polypeptide of the present invention by culturing

the transformed cell above in a medium; and

recovering said polypeptide from the medium and/or a cell extract of the cultured transformed cell.

10. The present invention still further provides a method for producing Gb/CD77 comprising the steps of:

exposing the polypeptide according to any one of claims 1 to 3, or a cultured product of the transformed cell according to claim 8, to lactosylceramide, to cause thereby enzymatic reaction; and

recovering Gb3/CD77.

The present invention still further provides a method for producing a glycolipid as represented by the following formula (1) comprising the steps of:

exposing the polypeptide of the present invention, or a cultured product of the transformed cell above, to galactosylceramide, to cause thereby enzymatic reaction; and

recovering the glycolipid represented by the following formula (1):

Galα1→4Gal-Cer (1)

wherein Gal represents a galactose residue, Cer represents a ceramide residue and α1→4 represents an α1-4 glycosidic linkage.

In the present invention, the enzyme having an activity to transfer a galactose residue from a galactose donor to C4 position of galactose residue of lactosylceramide or galactosylceramide which serves as an acceptor, will be called "α1,4-galactosyltransferase." Further, the activity of the enzyme to transfer a galactose residue from a galactose donor to C4 position of galactose residue of

lactosylceramide or galactosylceramide which serves as an acceptor, will be called "α1,4-galactosyltransferase activity."

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 shows flow cytometry indicating the expression of Gb3/CD77 by L cells. The left diagram relates to L cells transfected with pCDM8 while the right diagram to L cells transfected with pVTR1/CDM8. The thick line indicates the result of cells stained with mAb38.13 and FITC-conjugated rabbit anti-rat IgG (secondary antibodies) while the thin line the result of cells stained only with the secondary antibodies (control).

Fig. 2 shows TLC charts of glycolipids extracted from cells transiently transfected with α1,4 Gal-T gene.

A: TLC of glycolipids extracted from L cells transfected with pCDM8 (VC) or pVTR1/CDM8 (TF). RBC represents neutral glycolipids extracted from human B red blood cells.

B: TLC immunostaining of Gb3/CD77 by mAb38.13.

Fig. 3 shows the hydropathy plot of a polypeptide of the present invention.

Fig. 4 shows the α1,4 Gal-T activity in the extracts of transient transfectants of pVTR1.

A: α1,4 Gal-T activity when LacCer was used as an acceptor.

B: α1,4 Gal-T activity when various acceptors were used. PG represents paragloboside.

Fig. 5 shows the result of northern blotting of α1,4 Gal-T gene.

A: the upper columns show the results of hybridization with a ^{32}P-labeled probe derived from pVTR1, while the lower columns show the results of hybridization of the same membranes as in A with a β-actin cDNA probe(control).

B: the expression levels of mRNA of α1,4 Gal-T gene were compared among various human tissues. The ordinate represents the percentage of the expression level of a given tissue with respect to the level of heart after correction with the control.

Fig. 6 shows flowcytometry of stable transfectant cells. The left diagram relates to cells transfected with pSV2neo while the right diagram to cells transfected with pVTR1 and pSV2neo. The thin line indicates the number of cells stained with mAb38.13 and FITC-conjugated rabbit anti-rat IgG (secondary antibodies) while the thick line the number of cells stained only with the secondary antibodies (control).

Fig. 7 shows the results of MTT assay of L-neo and L-VTR1. The left graph shows the result of L-neo while the right one the result of L-VTR1.

Fig. 8 shows the effect of vero toxins on the cell growth.

Fig. 9 shows an electrophoresis indicating the result of DNA fragmentation assay.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

The mode for carrying out the present invention is described below.

<1> The polypeptide of the present invention

The polypeptide of the present invention is a polypeptide of (a) or (b) below:

(a) a polypeptide consisting of an amino acid sequence represented by the amino acid Nos. 46-353 in SEQ ID NO: 2; or

(b) a polypeptide which comprises an amino acid sequence including substitution, deletion, insertion or transposition of one or few amino acids in the amino acid sequence of (a) and which has an enzymatic activity to transfer a galactose residue from a galactose donor to C4 position of galactose residue of lactosylceramide or galactosylceramide which serves as an acceptor.

The above polypeptides contain at least a catalytic domain of α1,4-galactosyltransferase as will be described later. α1,4-galactosyltransferase comprises, in order from its N-terminal, a cytoplasmic domain, transmembrane domain, and catalytic domain. The polypeptides described above in (a′) and (b′) comprise at least the transmembrane and catalytic domains. The polypeptides described above in (a″) and (b″) comprise the cytoplasmic, transmembrane and catalytic domains. These peptides are all included in the polypeptides of the present invention.

An example of the amino acid sequence of a polypeptide of the present invention is represented in SEQ ID NO:2. In SEQ ID NO:2, amino acid Nos. 1-19 represents the cytoplasmic domain, Nos. 20-45 the transmembrane domain, and Nos. 46-353 the catalytic domain.

Among those polypeptides, the polypeptides (a), (a′) and (a″)

are preferred; the polypeptides (a′) and (a″) are more preferred; and the polypeptide (a″) is most preferred. However, any one of them may be used as long as it has an α1,4-galactosyltransferase activity.

In this specification, the term " a polypeptide which comprises an amino acid sequence including substitution, deletion, insertion or transposition of one or few amino acids and which has an enzymatic activity to transfer a galactose residue from a galactose donor to C4 position of galactose residue of lactosylceramide or galactosylceramide which serves as an acceptor″ means that, one or more amino acid residues of the polypeptide may be substituted, deleted, inserted, or transferred as long as such modification does not substantially affect the ability to the enzymatic activity (α1,4 Galactosyltransferase activity) of the polypeptide to transfer a galactose residue from a galactose donor to C4 position of galactose residue of lactosylceramide or galactosylceramide which serves as an acceptor.

Mutation such as substitution, deletion, insertion, or transposition of amino acid residues may occur in the amino acid sequence of the polypeptides existing in nature due to, for example, the modifying reaction of the biosynthesized polypeptides in the living organisms or during their purification as well as polymorphism and mutation of the DNAs encoding the polypeptides, nevertheless some of mutated polypeptides are known to have substantially the same physiological and biological activities as the intact polypeptides that have not been mutated. The polypeptide of the present invention

includes those having slightly different structures but not having a significant difference in the functions. The polypeptide of the present invention also includes those which have been artificially treated to have mutation as described above in the amino acid sequences. In this case, a further variety of mutants can be produced. For example, a polypeptide having a human interleukin 2 (IL-2) amino acid sequence, in which a cysteine residue has been replaced with a serine residue, is known to retain the interleukin 2 activities (Science 224, 1431 (1984)). Furthermore, a polypeptide of certain kind is known to have a peptide region that is not essential for exhibiting its activities. Examples of such polypeptides include a signal peptide contained in a polypeptide that is secreted extracellularly and a pro-sequence found in a precursor of protease, and the like. Most of these regions are removed after translation or upon conversion into an active form of the polypeptides. These polypeptides exist in different primary structures but finally have equivalent functions. Such polypeptides are also included in the polypeptide of the present invention.

The term "few amino acids" used herein means the number of amino acid residues that may be mutated to the extent that the enzymatic activities of the polypeptide of the present invention are not lost. For example, in a polypeptide consisting of 400 amino acid residues, about 2 to 20, preferably 2 to 10, more preferably 2 to 5 or less of amino acid residues may be mutated.

The α1,4-galactosyltransferase activity can be assayed by a

known method(ref.4). Specifically, the assay consists of using UDP-galactose (UDP-Gal) as a donor, and depending on the reaction where galactose is transferred by the enzyme to LacCer(acceptor). From above, it is obvious that any one skilled in the art could easily select substitution, deletion, insertion or transposition of one or more amino acid residues which does not substantially affect the enzymatic activity, using its α1,4-galactosyltransferase activity as an index.

The polypeptide of the present invention was obtained as follows :

cDNA of α1,4-galactosyltransferase was isolated from human melanoma cell line; the cDNA was expressed in mouse fibroblasts; and the peptide was identified and characterized, and its structure was determined. The polypeptide of the present invention may be obtained by expressing the DNA of the present invention as described later, in appropriate cells. The same polypeptides chemically synthesized are naturally included in the present invention. The method for producing the polypeptide of the present invention using the DNA of the present invention will be described later.

The polypeptide of the present invention is not necessarily a single polypeptide but may be a part of a fusion protein if necessary. A fusion protein comprising the polypeptide of the present invention and another polypeptide such as protein A may be cited as such an example.

The polypeptide of the present invention may consist of a

polypeptide alone, or contain a sugar chain or the like, as long as it has the enzymatic activity to transfer a galactose residue from a galactose donor to C4 position of galactose residue of lactosylceramide or galactosylceramide which serves as an acceptor.

<2> DNA of the present invention

The DNA of the present invention is a DNA encoding the polypeptide of the present invention as described above. The DNAs encoding the polypeptides described below in (a) and (b) may be cited as an example:

(a) a polypeptide consisting of an amino acid sequence represented by the amino acid Nos. 46-353 in SEQ ID NO: 2; or

(b) a polypeptide which comprises an amino acid sequence including substitution, deletion, insertion or transposition of one or few amino acids in the amino acid sequence of (a) and which has an enzymatic activity to transfer a galactose residue from a galactose donor to C4 position of galactose residue of lactosylceramide or galactosylceramide which serves as an acceptor.

Among the DNAs, the one coding for polypeptide (a) is more preferred.

The DNA encodes at least the catalytic domain of α1,4-galactosyltransferase, but the DNA of the present invention also includes DNAs encoding, in addition to above, the polypeptides including a transmembrane domain and/or cytoplasmic domain.

The DNA encoding the polypeptide (a) includes, for example, a DNA containing a nucleotide sequence represented by nucleotide Nos.

269-1192 in SEQ ID NO:1. The DNA encoding the polypeptide containing the transmembrane domain includes, for example, a DNA containing a nucleotide sequence represented by nucleotide Nos. 191-1192 in SEQ ID NO:1. The DNA encoding the polypeptide containing the cytoplasmic domain includes, for example, a DNA containing a nucleotide sequence represented by nucleotide Nos. 134-1192 in SEQ ID NO:1.

Furthermore the DNA comprising a nucleotide sequence represented by SEQ ID NO:1 has been derived from human originally. As a matter of course, however, the DNA of the present invention is not limited to any source, and includes those that are produced by genetic engineering procedure or chemical synthesis.

Furthermore, any one ordinarily skilled in the art would readily understand that the DNA of the present invention includes DNAs having nucleotide sequences different from what is described above due to degeneracy of the genetic codes.

The DNA of the present invention also includes DNA or RNA complementary to the DNA of the present invention. Furthermore, the DNA of the present invention may be either a single-stranded coding chain encoding the polypeptide of the present invention or a double-stranded chain consisting of the above single-stranded chain and a DNA or an RNA having a complementary nucleotide sequence thereto.

The DNA of the present invention was obtained by expression cloning as will be described later. However, since the nucleotide sequence of the DNA of the present invention was determined, it will be possible to isolate the same DNA from human-derived mRNA or cDNA,

or a chromosomal DNA through PCR with an oligonucleotide prepared from the nucleotide sequence thus determined to serve as a primer, or from a cDNA library or chromosomal DNA library through hybridization with an oligonucleotide prepared from the nucleotide sequence thus determined to serve as a probe.

The gene encoding the polypeptide of the present invention derived from a chromosome is expected to contain introns in the coding region. DNA fragments separated by introns are also included in the DNA of the present invention.

The DNA of the present invention may include DNAs, as long as they code for the polypeptides having an enzymatic activity to transfer a galactose residue from a galactose donor to C4 position of galactose residue of lactosylceramide or galactosylceramide which serves as an acceptor, hybridizable with a probe comprising a nucleotide sequence complimentary to the nucleotide sequence of SEQ. ID No:1, or to a nucleotide sequence represented by nucleotide Nos. 269-1292, nucleotide sequence represented by nucleotide Nos. 191-1292, or nucleotide sequence represented by nucleotide Nos. 134-1292 of SEQ ID No:1, or with a probe comprising a part of those nucleotide sequences, under a stringent condition. The "stringent condition" here refers to a condition under which a so-called specific hybrid is formed, but no non-specific hybrids are formed (see Sambrook, J. et al., Molecular Cloning A Laboratory Manual, Second Edition, Cold Spring Harbor Laboratory Press (1989)). The "stringent condition" may include for example subjecting a test DNA to a solution

containing 50% formamide, 4x SSC, 50mM HEPES(pH 7.0), 10x Denhardt's solution, and 100 μg/ml of sermon sperm DNA, allowing it to hybridize in the solution at 42°C, and washing the yield in 2x SSC and 0.1% SDS solution at room temperature, then in 0.1x SSC and 0.1% SDS solution at a temperature of 50°C or less.

Production of the polypeptide of the present invention may be achieved by cultivating transformed cells transfected with the DNA of the present invention on appropriate growth medium, thereby allowing the polypeptide of the present invention encoded by the DNA of the present invention to express itself, and by recovering the polypeptide thus expressed. The thus expressed polypeptide of the present invention can be extracted from a cultured product of the transformed cells (comprising both transformed cells and medium). However, if the polypeptide of the present invention is accumulated in the cytoplasm of transformed cells, or their membrane fraction, the polypeptide must be extracted from the transformed cells. Or, if the polypeptide is accumulated in medium, it must be extracted from medium. Or, if use of the transformed cells in which the polypeptide is expressed is desired, the transformed cells themselves, or their processed products may be used intact, or after they have been bound to an appropriate solid phase, or covered with gel for solidification. The "transformed cell" includes not only transformed cell themselves but also extracts from them.

For the transfection of the DNA of the present invention into a host cell, it is only necessary to prepare a recombination vector

by inserting the DNA of the present invention into an appropriate vector, and to introduce the DNA of the present invention into a host cell through the recombination vector. The vector is preferably an expression vector.

The host cell is not limited to any specific cells, as long as they can fully play the function of the DNA of the present invention, or of the recombination vector containing the DNA of the present invention. Thus, it may include any animal cells, plant cells, micro-organisms (bacteria), or the like. Procaryotic cells such as *E. coli*, or eucaryotic cells such as mammalian cells may be exemplified. When a procaryotic cell such as *E. coli* is used, addition of sugar chain does not occur to the polypeptide produced as by expression of the DNA of the present invention, then the polypeptide of the present invention having no sugar chain can be obtained. When eucaryotic cell such as a mammalian cell is used, sugar chain may add to the polypeptide produced by expression of the DNA of the present invention, then the form of the polypeptide of the present invention comprising sugar chain can be obtained.

Specifically, the host cell to transfect with the DNA of the present invention may include for example L cells derived from mouse fibroblasts. Specifically, the vector may include pCDM8 or pcDNA3 expression vector (both available from Invitrogen). The culture medium and condition may be chosen appropriately according to a given host cell.

The DNA of the present invention may be expressed directly.

Alternatively, it may be expressed with another polypeptide as a fusion polypeptide. The full-length DNA of the present invention may be expressed. It may also be expressed in part as a partial peptide.

The method for introducing the DNA of the present invention may depend on transfection based for example on DEAE-dextran method.

Recovering the polypeptide of the present invention from a cultured product may be performed by known extraction and purification methods for polypeptides. The cultured product used herein includes the medium and the cells in the medium.

Extraction of the polypeptide of the present invention may be performed, for example, by a method using a nitrogen cavitation apparatus, extraction from the cells disrupted by homogenization, glass bead milling, sonic wave treatment, osmotic shock, freeze-thawing procedure or the like, extraction by using detergent, or combination of those methods.

When the DNA of the present invention encoding the polypeptide (a") is expressed in L cells, the polypeptide of the present invention is localized at the membrane fraction of the cell. When the DNA of the present invention is expressed as a fusion protein comprising a polypeptide of the present invention (or a part thereof), and another peptide, so as to be a soluble protein, that fusion protein may be present in the cytoplasm. When the DNA of the present invention is expressed as a fusion protein comprising the polypeptide of the present invention or a part thereof and a secretion signal, the

resulting protein may be secreted into medium. Isolation of DNA encoding a part of the polypeptide of the present invention may be achieved by preparing a primer previously designed to produce such a DNA, and applying the primer in PCR to human derived mRNA, cDNA library, or chromosomal DNA.

Specific examples of the method of purifying the polypeptide of the present invention extracted from the cells or medium include salting out with salt such as ammonium sulfate or sodium sulfate, centrifugation, dialysis, ultrafiltration, absorption chromatography, ion exchange chromatography, hydrophobic chromatography, reverse phase chromatography, gel filtration, gel permeation chromatography, affinity chromatography, electrophoresis, and any combination thereof.

It can be confirmed whether the polypeptide of the present invention has been produced or not by analyzing amino acid sequence, action, and substrate specificity of the purified polypeptide.

<3> Utilization of the polypeptide and DNA of the present invention

The polypeptide of the present invention can be utilized for the synthesis of globo-series glycolipids, for example, for the synthesis of Gb3/CD77 by exposing the polypeptide of the present invention or a cultured product of the transformed cells trensfected with the DNA of the present invention to lactosylceramide, thereby evoking enzymatic reaction. Also, it is possible to obtain a glycolipid as represented by the following formula (1) by exposing the polypeptide of the present invention or a cultured product of

the transformed cells transfected with the DNA of the present invention to galactosylceramide.

Gal $\alpha 1 \rightarrow$ 4Gal-Cer (1)

In this procedure, exposure to substrate may occur through contact with the transformed cells, if the polypeptide of the present invention is produced and accumulated in the cytoplasm or in the membrane fraction, or through contact with medium if the polypeptide is accumulated in medium. When the cells in which the polypeptide of the present invention has been expressed are utilized, exposure to substrate may occur through direct contact with the cells themselves, or extracts therefrom, or immobilized extracts. The term "transformed cell" here includes not only transformed cells themselves but also extracts from them.

The polypeptide of the present invention is capable of specifically attaching a galactose residue to C4 position of galactose residue of lactosylceramide or galactosylceramide contained in a sugar chain of a glycoprotein. Further, the polypeptide of the present invention is utilized for selective synthesis of a sugar chain.

Although a number of members of β1,3-galactosyltransferases (β1,3Gal-Ts) or β1,4Gal-Ts have been identified(ref.27-30), this gene is the first and only α1,4Gal-T gene isolated so far. Moreover, no homologous genes to this gene were detected in the data base of *C. elegans* or *Drosophila melanogaster* genes, even though many β1,4 and β1,3Gal-T-related genes have been identified. These facts may

indicate that α1,4Gal-T gene evolved relatively later than other galactosyltransferase genes, and globo-series glycolipids synthesized through Gb3 are playing more precise roles compared to glycolipids of the other series.

Gb3/CD77 seems to be unusual because it can mediate various apoptotic signals in both normal cells and malignant tumor cells, even though it does not contain any cytoplasmic domain(ref.16 and 31). The observed rapid death of $CD77^+$ BC B cells *in vitro* suggests that endogenous ligand molecules interact with Gb3/CD77 to bring about the physiologic selection of immature B cells(ref.11 and 32). Furthermore, the capability of B subunit of VT to induce apoptosis of Gb3/$CD77^+$ cells(ref.16) strongly encourages the investigation of Gb3/CD77-associating cytoplasmic molecules(ref.31). Investigations of these ligands and signal transducers relevant to Gb3/CD77 might contribute to further understanding of the B cell selection and of the pathogenesis of hemolytic uremic syndrome caused by *E.coli* O157 infection. In particular, the tissue specificity of the syndrome such as renal failure, hemolysis and neurological disorders, might be clarified by gene manipulation of the cloned Gb3/CD77 synthase.

Furthermore, it has recently been reported that Gb3/CD77 and ganglioside GM3 may function as alternative cofactors for the entry of human immunodeficiency virus type 1 (HIV-1) in CD4-induced interactions between gp120 and glycosphingolipid microdomains(ref.33 and 34). If this is the case, Gb3/CD77 may be

a receptor not only for bacterial toxins but for viruses, and the regulation of Gb3/CD77 expression could be a key target for the therapeutic approaches of viral infections such as HIV-1.

Further, the expression of Gb3/CD77 in the kidney has been thought to be related with the development of hemolytic uremic syndrome (HUS). Furthermore, Fabry's disease is known as a disease in which Gb3/CD77 accumulates in the kidney, heart, brain and vasculature. From above, the polypeptide and DNA of the present invention, and the method of the present invention for producing Gb3/CD77 or a glycolipid may serve as a therapeutic tool, diagnostic tool or research tool for the treatment of diseases caused by the abnormal expression of Gb3/CD77 as described above.

(Example)

The present invention will be described more in detail below by means of examples.

(Example 1) Isolation of cDNA for α1,4-Galactosyltransferase (α1,4 Gal-T).

<1> Preparation of a cDNA library from a human melanoma cell strain, and cloning of α1,4 Gal-T cDNA.

A cDNA was prepared from poly(A^+) RNA of a human melanoma cell line SK-MEL-37 as described(ref.17). The cDNA library was constructed by inserting the cDNA into a vector plasmid pCDM8 (Invitrogen). The library contained 5 x 10^6 independent colonies. The strain of bacteria was *E. coli* MC1061/P3 (ref.18).

Since the SK-MEL-37 cell line does not express Gb3/CD77 on its

surface, it highly efficiently expresses α1,4 Gal-T, and thus the cDNA library prepared from this cell line is excellent for the present purpose.

Plasmids of the cDNA library were transfected into a mouse fibroblast L cells together with pdl3027 (polyoma T gene, provided by Dr. C. Basilico at New York University, New York) using DEAE-dextran as described (ref.18). L cells express a large amount of LacCer although they have no α1,4 Gal-T activity nor Gb3/CD77 expression(ref.19). The L cells, because of these characteristics, served as an excellent host in the cloning of cDNA for α1,4 Gal-T. The L cell was kindly provided by Dr. A. P. Albino at Sloan-Kettering Cancer Center, New York, and was maintained in Dulbecco's modified Eagle's minimal essential medium (DMEM) containing 7.5 % of fetal bovine serum (FCS).

After 48 h, the transfected cells were detached and incubated with a rat monoclonal antibody (mAb) 38.13 (ref.6) on ice for 45 min. After washing, cells were plated on dishes coated with rabbit anti-rat IgM (ZYMED) as described (ref.17). Plasmid DNA was rescued from the panned cells by preparing Hirt extracts, and transformed into MC1061/P3. The same procedure was repeated 5 times. The plasmid DNA was collected from the transformed cells.

Using microscale transfection of L cell and immunofluorescence assay, cDNA clones that determined the Gb3/CD77 expression were isolated. Cell surface expression of Gb3/CD77 was analyzed by flow cytometry (Becton Dickinson) as described(ref.19). MAbs 38.13 or

TU-1 (23) were used with FITC-conjugated rabbit anti-rat IgG or anti-mouse IgM (ZYMED), respectively. As a result, two clones showing positive reactions were successfully isolated. As described later, the two clones are essentially similar in nature, and thus one of them is called pVTR1, and further analysis was performed on that one.

Fig. 1 shows the results of flow cytometry of L cells transfected with the plasmid pVTR1 or the plasmid pCDM8 (containing no target sequence to serve as control). It is obvious from this that the cells transfected with pVTR1 express Gb3/CD77 while those transfected with pCDM8 alone do not express Gb3/CD77. It was thus demonstrated that α1,4 Gal-T cDNA inserted to pVTR1 is involved in the synthesis of Gb3/CD77.

<2> Extraction of glycolipids from the transformed cells, and identification of Gb3/CD77

Glycolipids were extracted as described(ref.21). Briefly, glycolipids were extracted from about 400 μl of packed cells using chloroform/methanol (2:1, 1:1, 1:2) sequentially. TLC was performed on a high performance TLC plates (MERCK, Darmstadt) using the solvent system chloroform:methanol:0.22 % $CaCl_2$ (60: 35:8) and sprayed by orcinol. For standards, bovine brain ganglioside mixture (Wako, Tokyo), neutral glycolipids from human erythrocytes, and Gb3 (Sigma) were used.

Glycosphingolipids extracted from the transformed cells showed definite Gb3 bands in TLC, although the transformed cells with pCDM8

alone showed no Gb3 band (Fig. 2A), suggesting that the cloned pVTR1 derived from α1,4Gal-T gene.

The identity of Gb3/CD77 was confirmed by TLC-immunostaining using an aluminum-backed silica plate (MERCK) as described(ref.21). After TLC, the plate was blotted onto PVDF membrane as described(ref.22). After blocking, the plate was incubated with mAb, then antibody binding was detected with ABC kit (Vector Laboratories, Burlingame, CA) and Konica Immunostaining HRP-1000 (Konica, Tokyo). This TLC-immunostaining revealed strong bands of Gb3 only in the extracts from the cDNA transfected cells (Fig. 2B).

<3> Nucleotide sequencing of gene for α1,4 Gal-T

The nucleotide sequence of the cDNA clone which was confirmed to express Gb3/CD77 as described above was determined by dideoxynucleotide termination sequencing using the PRISM dye terminator cycle sequencing kit and model 310 DNA sequencer (Applied Biosystems). The sequencing showed that the two clones are essentially the same in sequence. Accordingly, one of them was selected for subsequent analysis, and named pVTR1. The nucleotide sequence of cDNA in pVTR1 is shown by SEQ ID NO:1. The amino acid sequence encoded by this nucleotide sequence is shown by SEQ ID NOs:1 and 2.

The initiation codon is embedded within a sequence similar to the Kozak consensus initiation sequence(ref. 24 and 25). This open reading frame predicts a 353-amino acid protein with a molecular mass of 40,498 daltons.

Nucleotide and amino acid sequence homology search was carried out using the internet program BLAST (National Center for Biotechnology Information). However, no cDNA or protein having a high homology with these sequences was found in the database.

Amino acid sequence and hydropathy analyses (35) were performed with a software GENETYX-MAC version 8.0 (Software Development, Tokyo)(Fig. 3). A single hydrophobic segment with 26 amino acids was present near the amino terminus (amino acid Nos. 20-45 in SEQ ID NO: 2). This putative signal anchor sequence would place 19 residues within the cytoplasm and 308 amino acids within the Golgi lumen.

The presence of two potential *N*-glycosylation sites are indicated (amino acid sequence Nos. 121-123 and 203-205 in SEQ ID NO: 2). Relatively high frequency of proline (10/31) was detected at the C'-side of the transmembrane domain.

(Example 2) Characterization and Production of α1,4 Gal-T

<1> Enzyme assay of α1,4 Gal-T

Membrane fractions were prepared as described(ref.19) from L cells transfected with the gene for α1,4 Gal-T as obtained in Example 1. The enzyme activity of α1,4 Gal-T in the membrane fraction was measured as descibed previously(ref.4). The reaction mixture for the assay contained the following in a volume of 50 μl : 50 mM sodium cacodylate-HCl (pH 6.0), 10 mM $MgCl_2$, 5 mM galactonolactone (Sigma), 0.3 % Triton X-100 (Sigma), 0.4 mM (LacCer), 2.9 mM phosphatidylglycerol (Sigma), 0.2 mM UDP-Gal (Sigma), UDP-[^{14}C] Gal

(2.5×10^5 dpm) (NEN), and membrane fraction containing 50 μg protein. The protein concentration was determined by Lowry's methods(ref.20). The products was isolated by a C_{18} Sep-Pak cartridge (Waters, Milford, MA) and analyzed by thin layer chromatography (TLC) and autoradiography using a Bio-Imaging Analyzer BAS2000 (Fuji Film, Tokyo). The results are shown in Fig. 4A.

L cells transfected with pVTR1/CDM8 showed high Gb3 synthase activity (7,012 units, pmol/h/mg of protein) when LacCer was used as an acceptor. On the other hand, L cells transfected with pCDM8 alone were completely negative. Thus, this cDNA determined α1,4Gal-T activity and the surface expression of Gb3/CD77, indicating that this cDNA encodes the Gb3/CD77 synthase.

Enzyme activity toward other potential acceptors was also examined (Fig. 4B). None of the acceptors examined except LacCer and galactosylceramide showed significant levels of [^{14}C]galactose incorporation (Fig. 4B). Km values for these two substrates were 54.5 μM (LacCer) and 132 μM (galactosylceramide). The P1 antigen in the P blood group system is also formed by α1,4 galactose transfer (acting on paragloboside(PG)), but it was confirmed that this enzyme is not responsible for the synthesis of P1 antigen (Fig. 4B).

<u>(Example 3) Expression Analysis of α1,4 Gal-T Gene in Various Tissues (Northern blotting)</u>

Multiple Choice Northern Blots menbranes (OriGene Technologies, Rockville, MA) were used. They were hybridized with [^{32}P]dCTP-labeled cDNA probe of pVTR1 or control β-actin as described (ref.18

and 19). The relative expression levels of mRNA of α1,4Gal-T gene among human tissues measured by Bio-Imaging Analyzer BAS2000 (Fuji Film) are presented as a percentage of the value of heart after correction with the control. Expression levels of the α1,4Gal-T gene in various human tissues were examined by Northern blotting. Among tissues examined, heart, kidney, spleen, liver, testis and placenta strongly expressed the gene (Fig. 5).

(Example 4) Stable Transfection of Cells with pVTR1 Plasmid

To prepare stable transformants, pVTR1 and pSV2neo were co-transfected into L cells using Lipofection kit (TOYOBO, Tokyo, Japan). To select transformants, the cells were cultured in DMEM containing FCS (7.5 %) and G418 (300 μg/ml). G481 is inactivated by 3'-O-aminoglycoside phosphotransferase encoded by the neo gene.

G418-resistant cells were cloned by limiting dilution. Clones transfected with pSV2neo alone were prepared for control. These cells were incubated together with mAb38.13, followed by addition of FITC-conjugated rabbit anti-rat IgG for reaction, and the resulting cells were subjected to flow cytometry in the same manner as described above. The results showed that the cells transfected with pVTR1 and pSV2neo(L-VTR1) strongly expressed Gb3/CD77 while those transfected with pSV2neo alone(L-neo) did not express Gb3/CD77 (Fig. 6).

(Example 5) Reaction of Transformed Cells to Verotoxins

<1> MTT assay

To compare the reactions of L-VTR1 and L-neo to VTs, MTT assay

was performed using cells prepared in 48 well plates (1 x 10^4 cells/well) and cultured in the presence of VT1 or VT2. The assay was performed by triplicated samples. To quantify the cell proliferation, 50 μl of 5 mg/ml of MTT (Sigma) in PBS was added to each well.

After incubation for 5 h at 37°C, the supernatants were aspirated and 100 μl of n-propylalchohol containing 0.1 % NP40 and 4 mM HCl was added. The color reaction was quantitated using automatic plate reader IMMUNO-MINI NJ-2300 (Nihon InterMed, Tokyo, Japan) at 590 nm with a reference filter of 620 nm.

L-VTR1 in VT (+) medium showed marked growth suppression compared to that cultured in the absence of VT, while L-neo showed no effects of VT (Fig. 7). MTT assay of L-VTR1 and L-neo after the exposure to various concentrations of VTs revealed marked growth suppression of L-VTR1 even at 0.01 ng/ml, but not of L-neo (Fig. 8).

<2> DNA Fragmentation Assay

DNA fragmentation assay was performed to determine the mechanism responsible for the death of L-VTR1 treated with VTs. Cells were cultured in the presence of VT2 (200 ng/ml). After 24 h, cells were collected and the pellets were lysed in 100 μl of lysis buffer (10 mM Tris-HCl pH 7.4, 10 mM EDTA and 0.5 % Triton X-100) for 10 min at 4°C. After centrifugation, the supernatants were collected, and 2 μl of RNAse (10 mg/ml) and 2 μl of Proteinase K (10 mg/ml) were added. After incubation for 1 h at 37°C, the fragmented DNA was 2-propanol precipitated. Electrophoresis was conducted

using DNA derived from 1.5 x 10^6 cells in 2 % agarose gel containing 0.2 μg/ml ethidium bromide in TEA buffer.

Agarose gel electrophoresis of cytoplasmic DNA extracted from L-VTR1 revealed a clear pattern of DNA fragmentation characteristic of apoptosis (Fig. 9). In contrast, the L-neo sample did not show any ladder formation. Thus, it was confirmed that Gb3/CD77 generated by the cDNA serves as a functional receptor for VTs.

The present invention provides α1,4-galactosyltransferase, and DNA encoding thereof. That enzyme can be utilized for the production of globo-series glycolipids such as Gb3/CD77.

Further, the DNA is useful for production of the above described enzyme, or serves as a therapeutic tool, diagnostic tool or research tool for the treatment of diseases caused by the abnormal expression of Gb3/CD77, or it may be useful for the treatment or diagnosis of diseases involved in the action of verotoxins.

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